Further Reading

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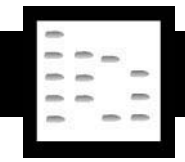
BIOMEDICAL APPLICATIONS



Gas Chromatography – Mass Spectrometry

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Introduction

The tremendous technological developments that have followed the initial interfacing of gas chromatographs with mass spectrometers together with the phenomenal advances in computerized data handling have provided an analytical technique that finds practically universal application. The biomedical sciences afford an enormous range of applications of this instrumentation where its full potential as a primary method for the separations and identification of extremely complex mixtures is clearly demonstrated.

The applications can be grouped into three broad categories based upon the nature of the analytes:

- Small volatile molecules, e.g. metabolites, xenobiotics (drugs, toxins, etc), food components
- Large labile molecular species, e.g. biomacromolecules and even whole cells
- Isotopomers (molecules differing only in isotopic composition), e.g. tracer studies, isotopic labelling, breath gas diagnostics, natural abundance studies

The analysis of small volatile molecules, perhaps after derivatization, is the major application area; particular biomedical applications in clinical chemistry and occupational hygiene are illustrated below. Others are exemplified under the headings sport, environment, food and forensics. The analysis of biomacromolecules and whole cells is another rapidly expanding field but using other mass spectrometric and separatory techniques (electrospray, atmospheric pressure and matrix assisted laser desorption ionization; see also LC–MS, CE–MS). Gas chromatography–mass spectrometry (GC–MS) of pyrolysates can provide information about otherwise intractible materials.

The elucidation of mechanisms and biochemical pathways using tracer and labelling techniques is an example of the third category of applications which is also a rapidly expanding area with the wider availability of stable as opposed to radioactive labelled compounds. The ability to separate and distinguish between components in a complex mixture that differ solely in their isotopic composition allows exogenous materials to be distinguished from endogenous species; it also provides a means of improving quantitation (via isotope dilution) and allows dynamic studies of metabolism or dysfunction to be undertaken. It is this latter area of GC–SIRMS (GC–stable isotope ratio MS) that will be emphasized.

Analysis of Small Volatile Molecules

Instrumentation

The majority of instruments utilize capillary columns thereby allowing relatively simple connection to the mass spectrometer. Earlier systems used packed columns that required some form of separator in order

to reduce the amount of carrier gas entering the mass spectrometer ionization chamber. A 5% poly(diphenyldimethylsiloxane) stationary phase is frequently used because of its wide general applicability and stability. Samples can be introduced using an autoinjector or manually by syringe; headspace gases, solutions in volatile solvents, solid-phase microextraction (SPME) systems and thermal desorption with cryofocussing can all be used. In some cases it is necessary or advisable to derivatize the sample in order to enhance its thermal stability.

Mass spectrometers of any type can be coupled, from huge magnetic sector machines to time-of-flight systems and the small 'bench-top' quadrupole or ion trap instruments. When the mass spectrometer is operated in positive ion mode with electron impact ionization at 70 eV spectra generated can be compared with those in the extensive databases organized by NIST and Wiley. Using computerized file-handling techniques, spectra can be compared very rapidly and a list of possible compounds can be compiled. The mass spectrometer can also be operated in negative ion mode which allows improved sensitivity with particular analytes especially with chemical ionization.

Applications

Figure 1 shows typical results from a GC–MS study using a bench-top quadrupole system. The sample was obtained by extraction of serum taken after administration of an oestradiol prodrug. The upper

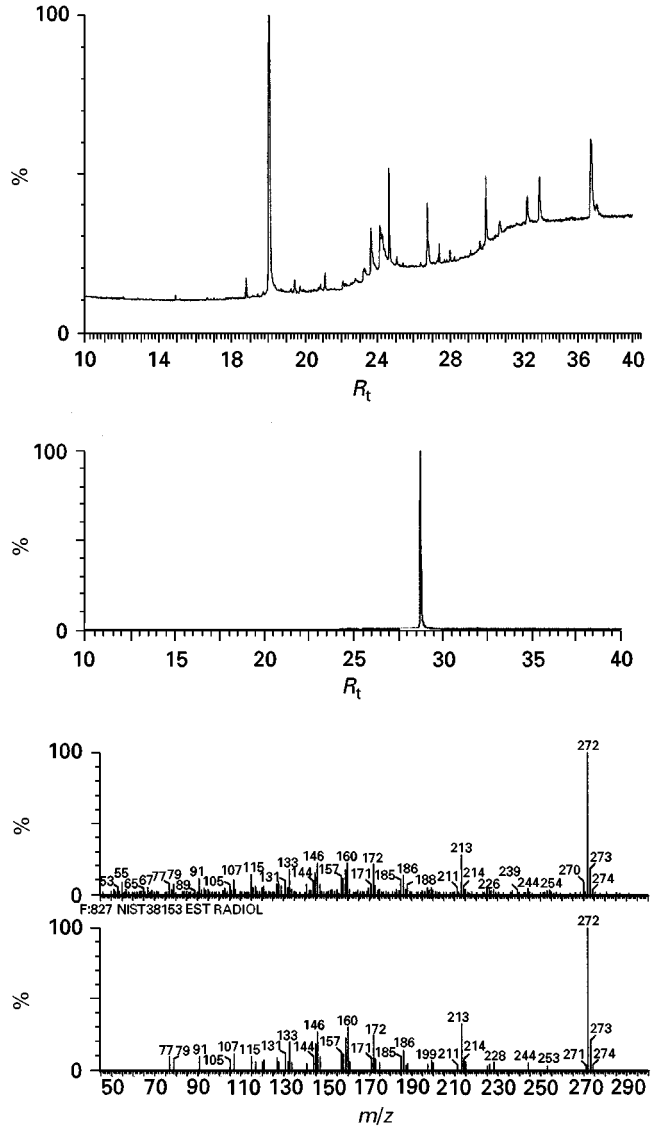

Figure 1 GC–MS of serum extract following oestradiol prodrug administration: (top) total ion chromatogram; (middle) mass chromatogram for $m/z = 272$; (bottom) mass spectrum for peak at $R_t = 28.76$ min and NIST library spectrum for oestradiol.

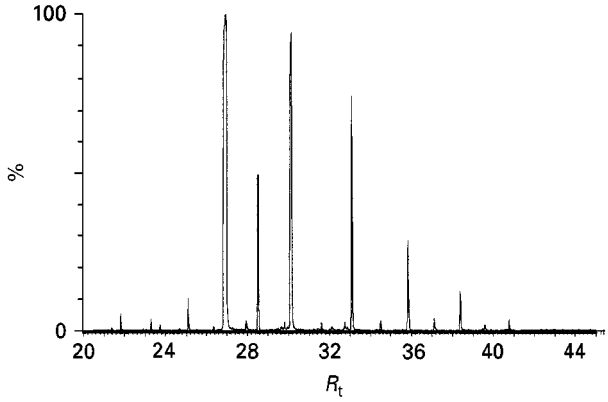

Figure 2 Mass chromatogram for $m/z = 74$ identifying FAMEs.

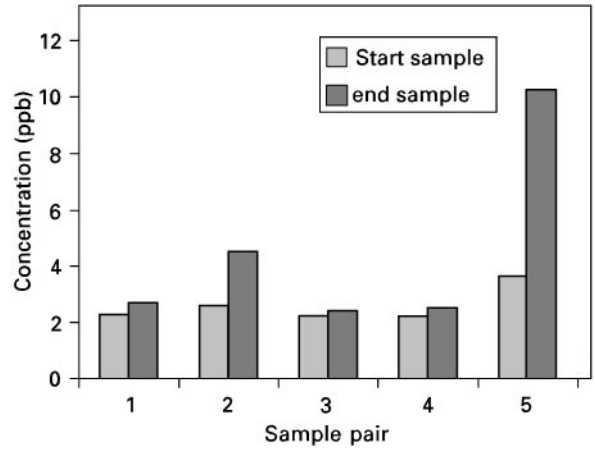

Figure 4 Urinary amine concentrations of paired samples.

trace shows the total ion chromatogram (TIC), the second trace shows the mass chromatogram for $m/z = 272$ which is diagnostic for oestradiol. The mass spectrum obtained for this component and the NIST library match are the lower traces.

Use of mass chromatograms of diagnostic ions allows facile recognition of homologous series such as fatty acid methyl esters (FAMEs). **Figure 2** shows the mass chromatogram for $m/z = 74$ for the FAME derivatives prepared from an archeological sample; this valuable information assisted in the interpretation of the pottery artifact.

The signal at $m/z = 74$ is due to methyl ethanoate formed in the mass spectrometer by a McLafferty rearrangement shown in **Figure 3**. This process can occur with any FAME having an available hydrogen atom at position 4 and thus provides a useful diagnostic ion.

Selected ion recording (SIR) measures the ion current for a restricted range of ions instead of the whole spectrum. This is of particular use in biomonitoring studies for example where the analyte is well characterized from the chromatographic and spectrometric point of view, affording improved sensitivity and precision.

Figure 4 is a graphical representation of the data from an occupational health study of urinary amines: paired samples of urine taken at the beginning and end of a working day were analysed for a particular aromatic amine. A protocol was used that freed the amine from excreted conjugates followed by extraction and derivatization. The graph emphasizes the difference between the amounts of amine in the paired samples; although the values were well below regulatory limits, those from the fifth pair led to a change in working practice for that donor.

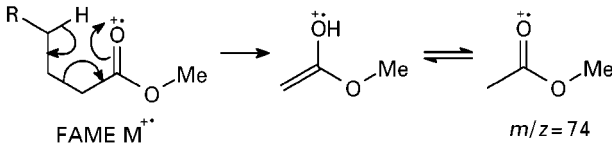

Figure 3 McLafferty rearrangement in a FAME $M^{+\cdot}$ to form $m/z = 74$.

Amines can be converted into perfluoroacyl derivatives which are more stable thermally and chemically; such derivatives give improved sensitivity in electron-capture detectors and this is also manifest in negative ion chemical ionization mass spectrometry. This approach can be adopted for the analysis of materials that form relatively stable gas phase anions in the mass spectrometer. **Figure 5** shows data from another typical example: prostanoids such as $PGF_{2\alpha}$ are converted into the t-butyldimethylsilyl ether/pentafluorobenzoyl ester derivatives. Under negative ion chemical ionization conditions using either methane or ammonia as reagent gas, the ester function is lost in a fragmentation reaction to form a fragment ion at $m/z = 695$ corresponding to the silyl ether carboxylate anion shown in the figure. This ion shows satellites due to silicon and carbon isotopes at 696 and 697 that are in accord with calculated distributions. Detection levels for this analyte are in the low pg μL^{-1} (ppm) range for full scan data and fg μL^{-1} (ppb) range with selected ion recording.

The final example of this first group of applications involving small volatile species crosses the boundary into analysis of large intractible materials and concerns a pyrolysis study. Occasionally there may be insufficient sample to carry out a normal extraction prior to GC–MS analysis, this is particularly so with conserved archeological material and microscopic biopsy samples. In such circumstances, microscale sealed vessel pyrolysis GC–MS can be applied: **Figure 6** shows the TIC obtained from a hair sample (2 mg) taken from an Egyptian mummy. The individual components of the pyrolysate can be identified

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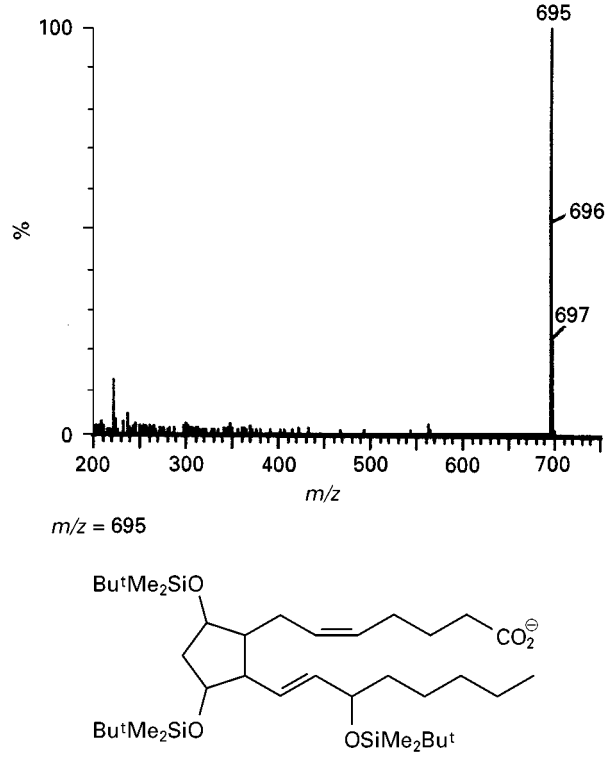

Figure 5 Mass spectrum of derivatized $PGF_{2\alpha}$ in negative ion chemical ionization and structure of anion corresponding to $m/z = 695$.

by comparison with library spectra and interpreted in terms of the mummification process.

This pyrolysis method can be applied to other complex analytes including whole cells in order to investigate occluded materials or for the identification of chemical modification of biopolymers such as starch.

The quantitative analysis of halogenated dibenzodioxins and -furans in biological (and environmental) matrices is a good example of the combination of high resolution GC (HRGC) and MS (HRMS) technologies using the isotopes of chlorine and carbon

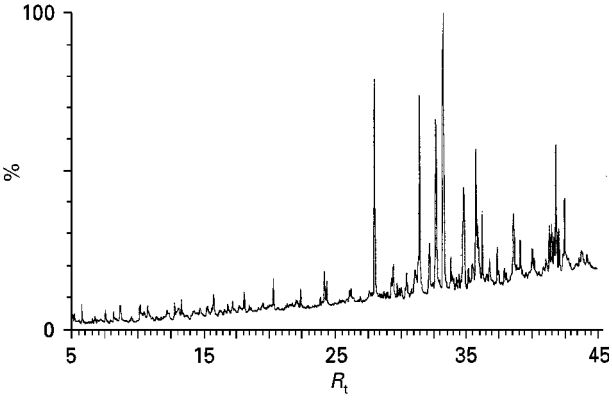

Figure 6 Total ion chromatogram from GC–MS of pyrolysis products from archaeological hair sample.

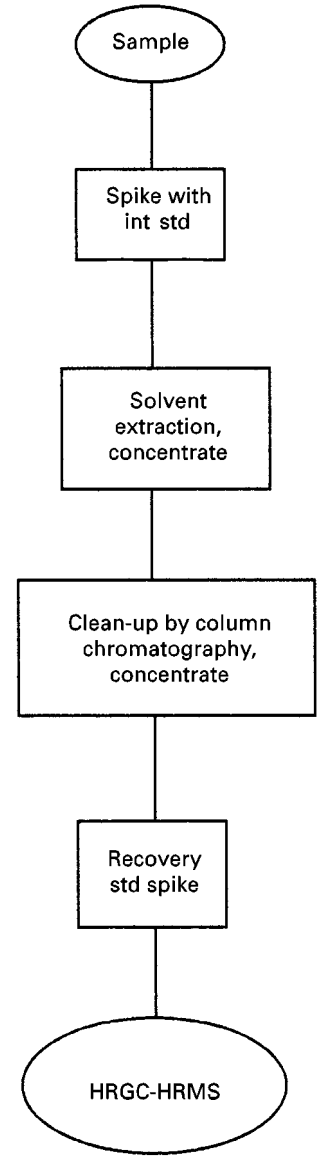

Figure 7 Protocol for pretreatment of dioxin/furan samples prior to HRGC–HRMS.

to facilitate identification and quantification. Prior to any instrumental analysis, the flesh, vegetation or other material has to be prepared according to a quality assured protocol that is summarized in **Figure 7**.

Intrinsic to the procedure is the use of standards: the first standard to be added is $^{13}C_{12}$-2,3,7,8-tetrachlorodibenzodioxin upon which quantitation is based. Another standard, $^{13}C_{12}$-1,2,3,4-tetrachlorodibenzodioxin is added after the chemical manipulation of the sample is complete and immediately prior to GC–MS analysis. Comparison of the signals from the two standards then allows the efficiency of the extraction process to be assessed. Typical data sets are shown in **Figures 8** and **9**: both show four traces, the upper two traces are the high resolution

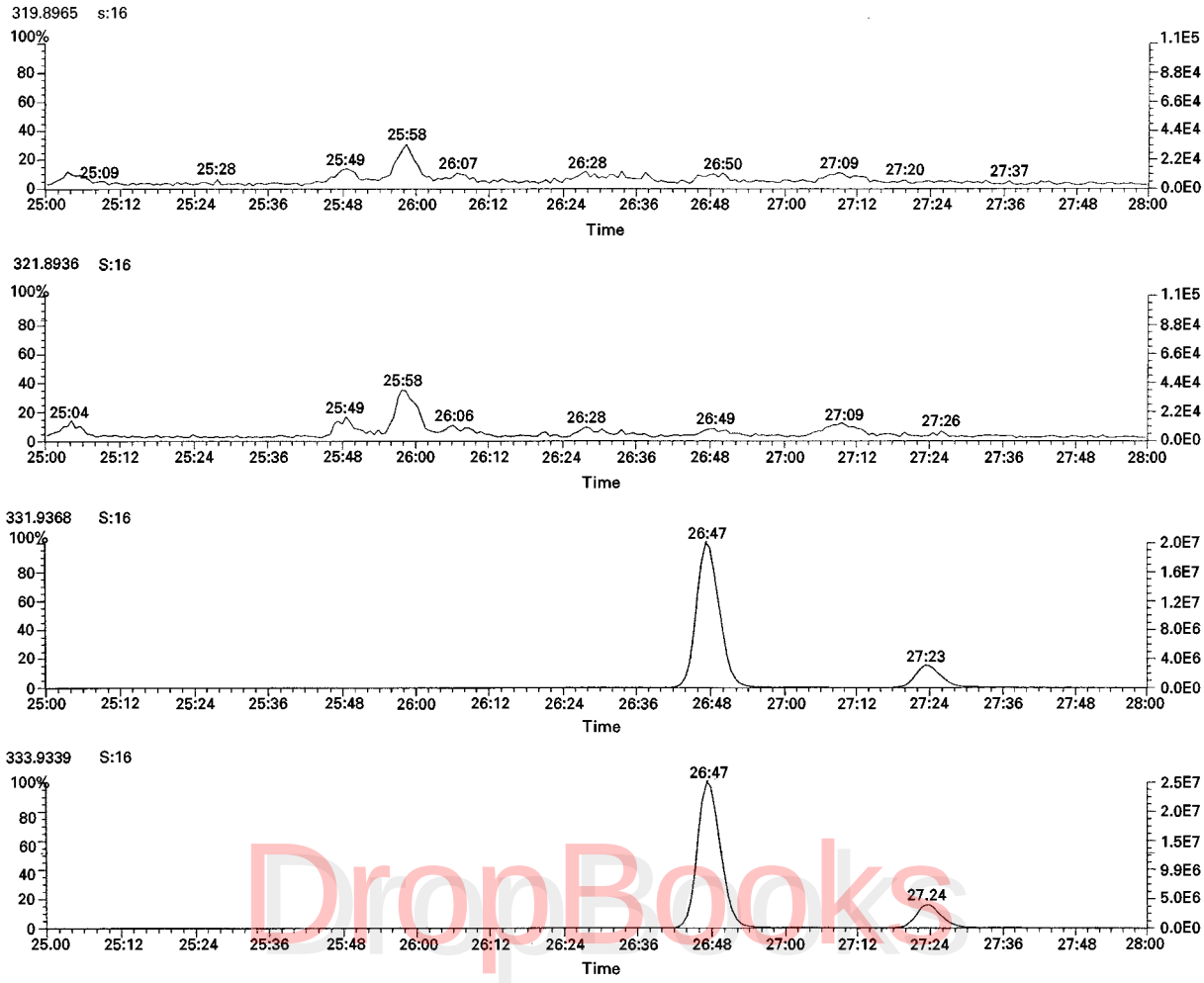

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Figure 8 HRGC–HRMS SIR data from dioxin/furan analysis of meat extract.

SIR data for $m/z = 319.8965$ and 321.8936 corresponding to the molecular ions of tetrachlorodibenzodioxins (TCDD), i.e. $C_{12}H_4(^{35}Cl_4)O_2$ and $C_{12}H_4(^{35}Cl_3{}^{37}Cl)O_2$ respectively. The lower two traces in each case correspond to the ions of $m/z = 331.9368$ and 333.9339 for the ^{13}C-isotopomers, i.e. $^{13}C_{12}H_4(^{35}Cl_4)O_2$ and $^{13}C_{12}H_4(^{35}Cl_3{}^{37}Cl)O_2$ for the two standards.

The data shown in Figure 8 represents TCDD levels below regulatory limits whereas those in Figure 9 (from an environmental sample) were significantly higher.

Analysis of Isotopomers

The above example shows the dual benefits of high resolution instrumentation and isotope dilution to effect precise quantification and identification. There are many other examples of this type of application that relies upon analysis of the intact analyte molecule in the mass spectrometer. The second group of applications follows a different approach in which isotopomeric components are initially separated then converted into light gases, carbon dioxide, hydrogen and nitrogen. It is these latter materials that are investigated in the isotope ratioing mass spectrometer whereby the ratios of carbon, hydrogen, oxygen and nitrogen isotopes are determined with much greater precision.

Instrumentation

The design of the mass spectrometer is extremely simple comprising an electron impact ionization source, a very stable magnetic analyser and triple Faraday cup collector (**Figure 10**A).

The combustion interface incorporates a furnace containing heated copper oxide which converts organic chemicals in the column effluent into carbon dioxide and water; a cold trap is used to remove the water vapour. Conversion of the individual components into the same chemical species such as carbon dioxide removes some of the variables that arise from

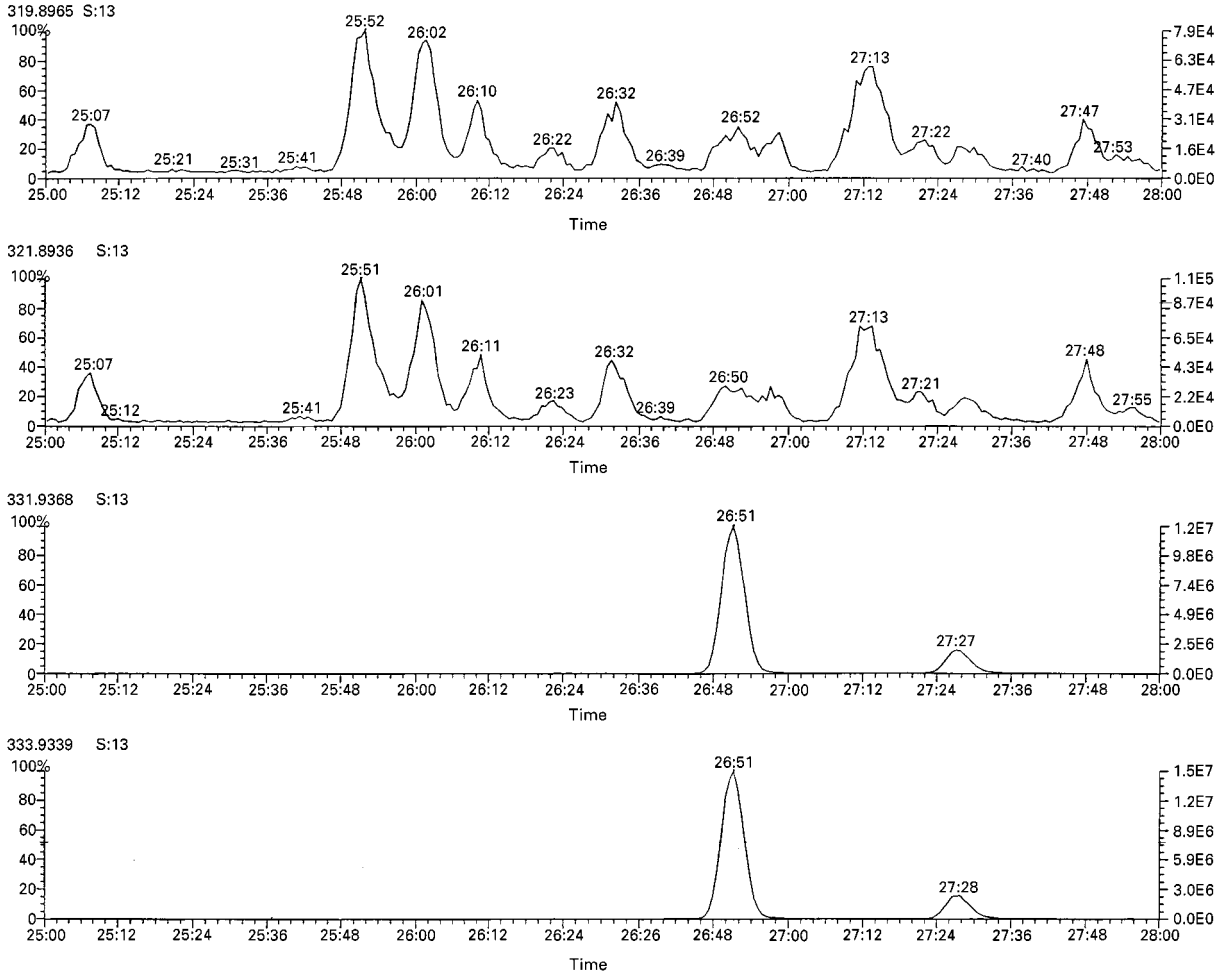

Figure 9 HRGC–HRMS SIR data from dioxin/furan analysis of an environmental sample.

different sample behaviour and isotopomeric distribution commonly observed in electron impact ionisation. Simultaneous recording of the ion beams at $m/z = 44, 45$ and 46 (corresponding to $^{12}C^{16}O_2$, $^{13}C^{16}O_2$ and $^{12}C^{16}O^{18}O$ respectively for carbon dioxide) using three separate Faraday collectors minimizes background signal fluctuation. These differences result in a dramatic improvement in the stability of the signal allowing determination of isotopic ratios at about 10^{-5} at%. **Figure 10**B shows a graphical output from the three collectors for three reference gas pulses and one sample.

The units used to express the relative difference in isotopic abundances are either atoms% (at%) or delta (δ, per mil or ‰).

$$\text{At\%} = \left(\frac{\text{no. of minor atoms}}{\text{no. of major atoms}}\right) \times 100$$

$$\delta = \frac{(\text{sample ratio} - \text{reference ratio})}{\text{reference ratio}} \times 1000$$

Applications

The use of stable isotopes such as ^{2}H, ^{13}C, ^{15}N or ^{18}O instead of the radioactive analogues removes all risks associated with radiation but the introduction of an isotopic substitution at the site of a rate-limiting reaction can introduce kinetic isotope effects. Thus an artificial change to the natural isotopic distribution could in theory alter the kinetics of the biochemical process and thereby affect the overall metabolic rate. Many theoretical calculations and experimental observations have been made: the greatest differences occur with hydrogen replacement with a maximum relative rate ratio (k_H/k_D) of 18. The differences with other isotopic replacements are much smaller: $^{12}C/^{13}C$ up to 1.25; $^{14}N/^{15}N$, 1.14; $^{16}O/^{18}O$, 1.119 and $^{32}S/^{34}S$, 1.05. This particular risk is however even smaller than that with the corresponding radiolabels whose values are $^{1}H/^{3}H$, 60 (max) and $^{12}C/^{14}C$, 1.5.

Breath Gas Testing Using $^{13}CO_2$

The basic principle is illustrated in **Figure 11**.

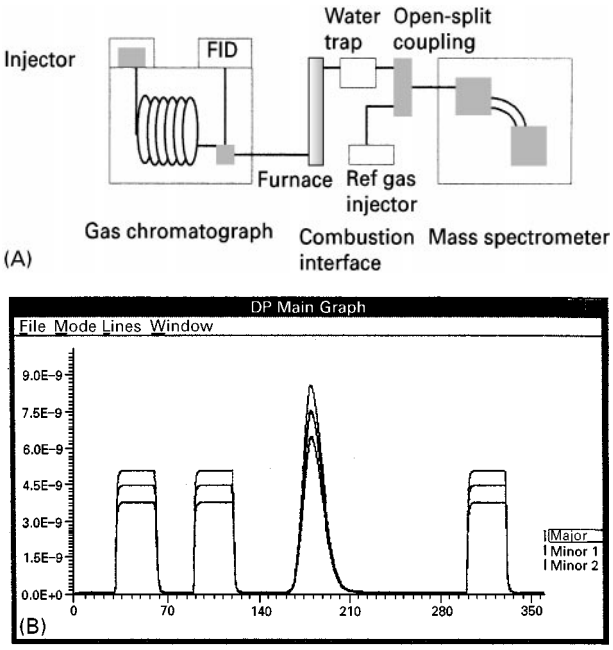

Figure 10 (A) Schematic representation of a GC–SIRMS system (B) Graphical output from SIRMS of two reference gas pulses, sample, reference gas pulse.

Oral administration of either a solution of the ^{13}C-labelled substrate in water or fruit juice or a suspension in a flavoured colloidal preparation is usually preferred to intravenous injection to emphasize the 'non-invasive' approach. Breath gas is the easiest means of sample collection. However, there are others including saliva, urine, faeces, milk, hair, nails or blood, gastric fluid or muscle. These latter requiring clinical intervention.

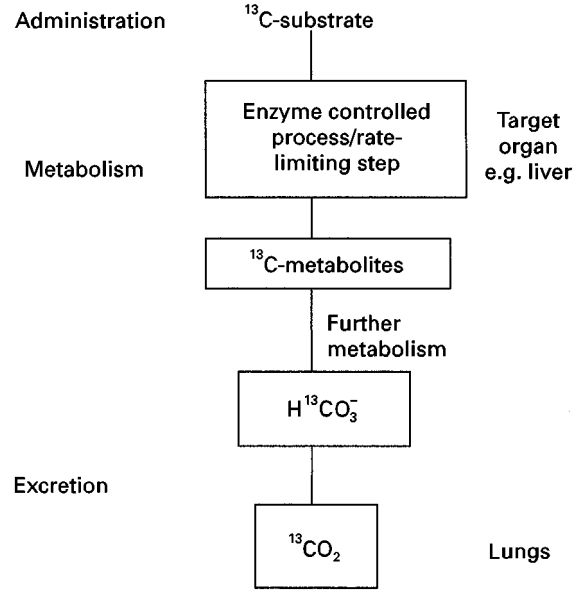

Figure 11 Schematic diagram of procedure for breath gas testing.

The ^{13}C-urea breath test for *Helicobacter pylori* A characteristic of this bacterium is its high urease activity; this is exploited by administering a solution of ^{13}C-urea (1 mg kg^{-1} body weight, ca. 75 mg for an average adult) and investigating the effect on the level of $^{13}CO_2$ in the breath after a short time interval of about 20–30 min. If the bacterium is present, the urea is converted into bicarbonate which appears in the breath as $^{13}CO_2$; in its absence the isotopic distribution remains unchanged (**Figure 12**).

Typical results from a group of untreated and treated patients are shown in **Figure 13**: differences between the pre- and post-breath samples (referring to ^{13}C-urea administration) of more than 3‰ are taken to indicate infection.

Other metabolic breath tests Introduction of a variety of ^{13}C-labelled substrates into a test meal that is ingested by the patient can be used to monitor particular metabolic functions *in vivo* without the need for invasive surgery and the collection of biopsy samples and subsequent *in vitro* biochemical investigation. Thus gastric emptying rates can be measured using octanoic acid labelled at position 1 with ^{13}C; abnormalities in the cytochrome P450 pathway in liver metabolism can be determined using a variety of substrates shown in **Figure 14** with typical data shown in **Figure 15**.

These data show that measurements taken after a time interval of one hour (after dosing with the labelled substrate) allow unambiguous detection of abnormality. A similar distinction is possible for lipid malabsorption using a range of ^{13}C-labelled triacyl glycerides. The results summarized in **Figure 16** are from a study of absorption of ^{13}C-dodecanoic acid introduced intraduodenally.

Tracer studies

The facile determination of carbon isotope ratios in specific compounds extends the scope of tracer studies to include assessment of metabolic rates for a wide variety of substrates. Thus administration of specifically labelled lipids, amino acids and carbohydrates followed by GC–SIRMS analysis of fractionated serum samples allows not only the identification of

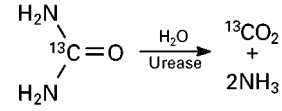

Figure 12 Chemical basis of the *Helicobacter pylori* breath test.

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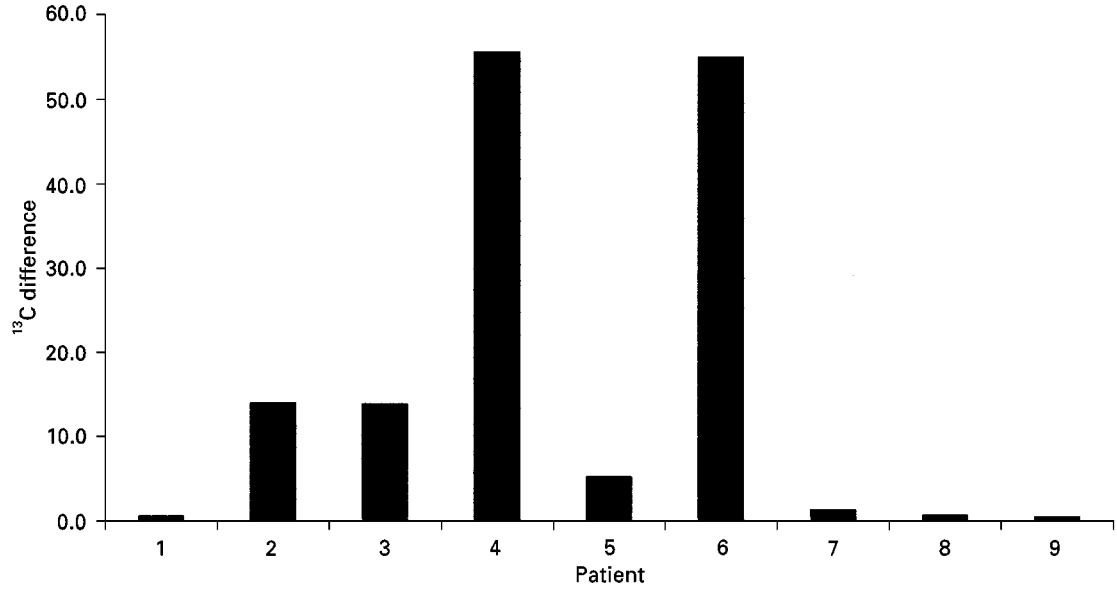

Figure 13 Typical results from *Helicobacter pylori* breath tests.

metabolic products but also the measurement of turnover rates of those substrates. The sensitivity of the system is such that measurements can be made using enriched substrates (as opposed to fully labelled compounds). **Figure 17** summarizes the results of a study using ^{13}C-enriched maize sugar administered at 1 g kg^{-1} body weight then timed blood samples purified and analysed for enrichment of plasma glucose by GC–SIRMS.

The data demonstrated that even at relatively low levels of enrichment, the appearance rate could be measured.

Natural abundance measurements

Primary and secondary kinetic isotope effects, although small, lead to fractionation of isotopomers and the observed natural variations in isotopic abundances. These temporal and geographical variations can be measured using SIRMS and used to detect adulteration and authenticity of foodstuffs and identify migration patterns of insects.

The variation in ^{13}C content of a variety of natural materials is summarized in **Figure 18**. Plants convert carbon dioxide into carbohydrates by two main photosynthetic mechanisms: the Calvin–Benson cycle or the Hatch–Slack pathway. Plants such as rice, wheat, potatoes, beet or brassicas utilize C_3 intermediates and result in depleted ^{13}C content (i.e. delta values are more negative) whereas maize, sugar cane and tropical fruits use C_4 intermediates that demonstrate higher ^{13}C content. The differences are large enough to be translated through the food web and be measured by GC–SIRMS.

The average δ-^{13}C value of honey, produced from flowers of C_3 plants by bees, is significantly lower than that of high fructose corn syrup (HFCS, produced from maize, a C_4 plant). Hence, it is possible not only to identify adulteration of honey with HFCS but also to determine its extent.

Many natural products, e.g. vanillin, are also available as synthetic products from chemical or biochemical syntheses; the distinction between natural, nature-identical and synthetic can be determined using GC-SIRMS. The natural product obtained by extraction has a generally higher δ-^{13}C value (ca. −20‰) compared to fossil fuel derived material (ca. −30‰) or fermentation product (ca. −35‰).

The method can be used to distinguish exogenous materials from their endogenous counterparts; this is particularly useful in the detection of anabolic steroids' administration in meat products, animals, racehorses or athletes where conventional GC–MS

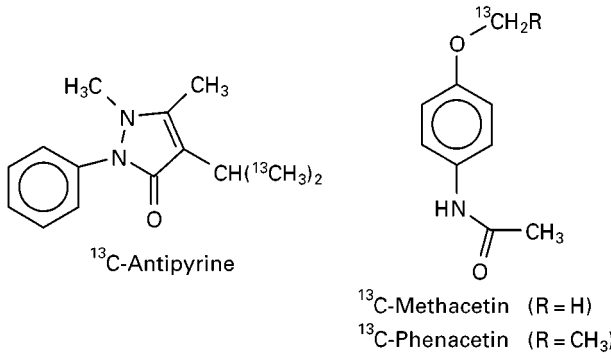

Figure 14 Substrates for breath tests of liver metabolism.

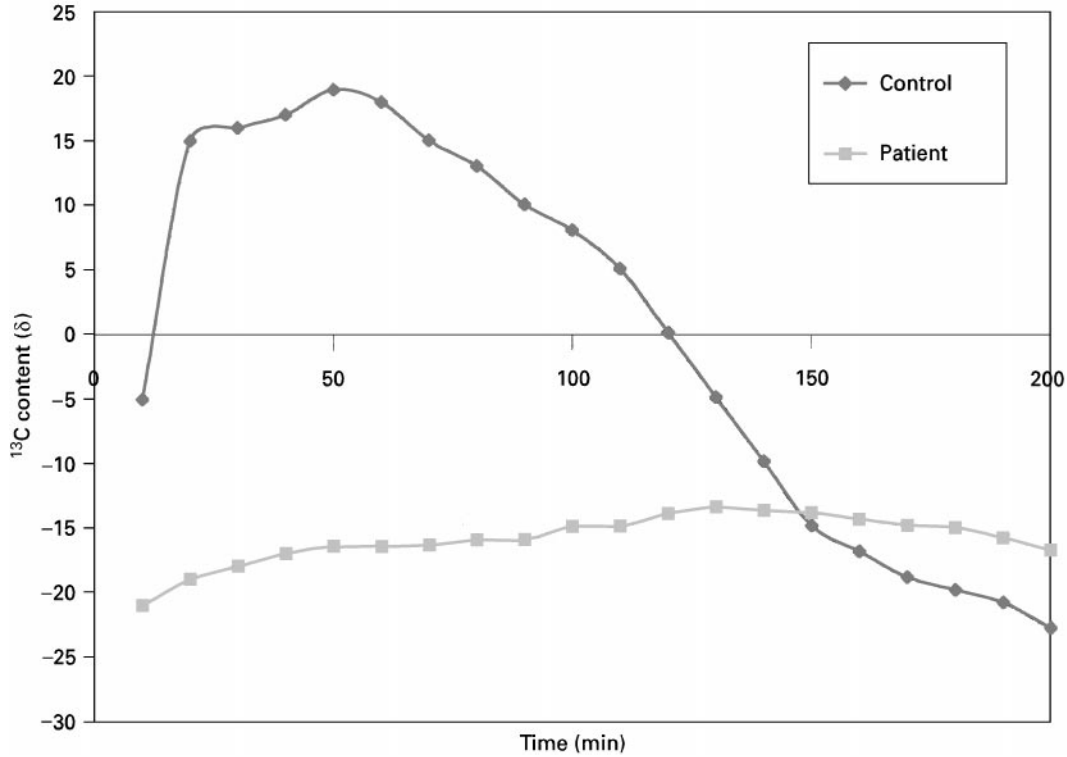

Figure 15 Comparison of data from patient and control for liver metabolism.

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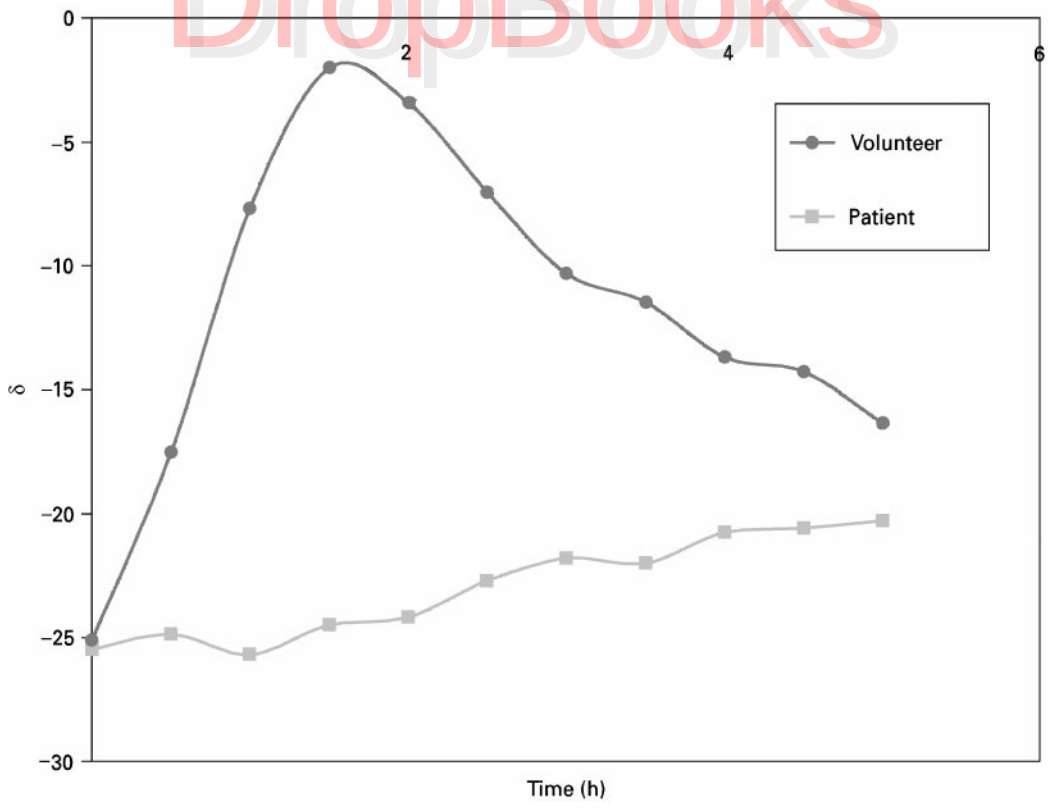

Figure 16 Appearance data for breath gas analysis following ^{13}C-dodecanoic acid administration in a study of lipid malabsorption.

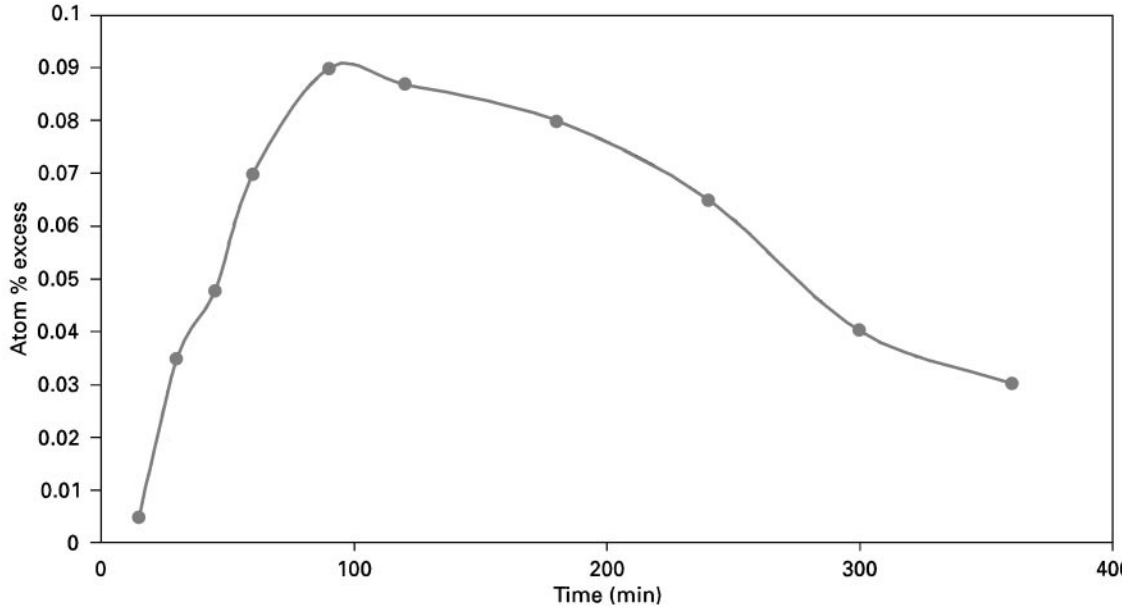

Figure 17 Appearance rates from a GC–SIRMS study of plasma glucose.

protocols have been shown to be inadequate. When the carbon isotope ratios for the major metabolites of testosterone, for example, are normalized to an endogenous reference compound such as cholesterol, the differences between testosterone-treated and untreated animals is statistically significant.

It is not only carbon that shows variability in its isotopic distribution; hydrogen and oxygen also show geographical variability that is correlated to rainfall latitude. Continuous-flow pyrolysis measurements of δD and δ^{18}O have been used to study animal migration patterns using a development of the technology described above. The use of helium as a carrier gas means that there is a large signal generated for He^+ at $m/z = 4$ and this needs to be totally resolved from the relatively small signal at $m/z = 3$ due to HD^+. This is further complicated by the presence of ions at $m/z = 3$ due to H_3^+ arising from an ion molecule reaction in the mass spectrometer source. This latter problem is overcome by application of a correction factor calculated from data from reference gas pulses.

The sample (e.g. insect wings, ca. 1 mg) is pyrolysed in a helium stream in a furnace containing quartz chips and nickel carbide at about 1000°C. The pyrolysis products, hydrogen, carbon monoxide and nitrogen, are separated using a packed column (1.5 m, molecular sieve 5 Å) then introduced into the mass spectrometer.

Reference pulses of hydrogen and carbon monoxide are introduced prior to the sample peaks and used to calculate the δD and δ^{18}O values for m/z 3/2 and m/z 30/28 and 29/28 (for carbon monoxide). A typical data set is:

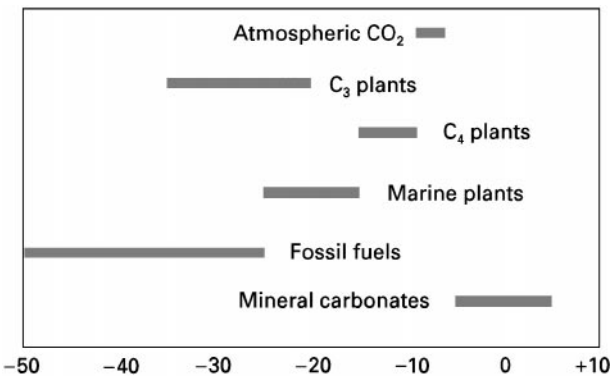

Figure 18 Natural variation in ^{13}C content (‰) of natural materials.

	Sample 1	Sample 2	Sample 3
Latitude	30°	40°	50°
δD precipitation	− 15‰	− 33‰	− 80‰
δD sample	− 78‰	− 92‰	− 125‰
δ^{18}O sample	+ 5.7‰	+ 3.8‰	+ 0.3‰

Conclusion

GC–MS will continue to provide much high quality and fundamental analytical information that will allow the biomedical sciences to develop along the avenues described above for some time. Improvements in the technology of extraction and sampling will allow progressively smaller samples to be used.

The availability of relatively inexpensive and safe isotopically labelled substrates and precursors will benefit tracer studies and allow improved precision in quantitative measurements of a wide variety of analytes. GC–SIRMS will facilitate dynamic studies of metabolism and migration. The capacity to distinguish exogenous from endogenous materials is bound to be a significant and expanding area of application that will find great use in the identification of abuse, adulteration and authenticity.

Acknowledgements

The following are acknowledged: Nick Ordsmith and Keith Hall from Hall Analytical Laboratories Ltd. Andy Phillips from Micromass Ltd. Nick Bukowski from ThermoQuest.

See also: **II/Chromatography: Gas:** Detectors: Mass Spectrometry. **III/Drugs of Abuse: Solid-Phase Extraction.**

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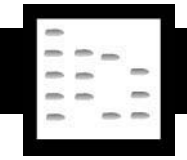
Thin-Layer (Planar) Chromatography

See **III / CLINICAL CHEMISTRY: THIN LAYER (PLANAR) CHROMATOGRAPHY**

BITUMENS: LIQUID CHROMATOGRAPHY



DropBooks

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Introduction

Great confusion exists in the literature on the definition of the term *bitumen*. It occurs in the earth's crust in various forms: firstly, in a dispersed state, in trace quantities; and secondly, as accumulations, where bitumen either impregnates the rock or occurs in a pure or nearly pure form. In the first half of the 20th century, the term bitumen was applied to crude oil and its natural derivatives (maltha, asphalt, ozokerite). In this original meaning, bitumens are called naphthides. The term bitumen is also used for asphalts and asphalt-like manufactured substances utilized in road construction, including products from the processing of coals and/or oil shales. In crude oils, in distillate cuts beginning with kerosene, and in distillation residues, there is a group of high molecular weight heteroorganic compounds that are lumped under the name of resins and asphaltenes. Their content may be as large as 40% in heavy crude oils. Carbon and hydrogen constitute 80–95% of the resin and asphaltene molecules, with oxygen always present. Sulfur, nitrogen and metals are also usually present. The content of resins in crude oil (2–40%) exceeds by far (from 3 to 40 times) the content of asphaltenes (trace to 6%; usually $<1\%$). Carboids and carbenes, which resemble asphaltenes, differ from them by having a higher oxygen content. However, they are absent in crude oils and distillate cuts. In small quantities, they are found in residues from vacuum distillation, in cracked tars, and in native and petroleum asphalts.

Bitumens and the related heat-treated asphalt are a very complex mixture of compounds with a wide range of molecular weights, which are difficult to isolate into classes of pure compounds. Resins are semi-liquid (sometimes almost solid) dark brown to black substances which have a specific gravity around 1 and a molecular weight of 500–2000 (usually 600–1000). Asphaltenes, on the other hand, are dark, amorphous powders and have a specific gravity greater than 1 and molecular weights of 1000–10 000 (average 5000). Using various methods, the observed molecular weights suggest that asphaltenes form molecular aggregates or colloids, even in dilute